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Evidence for a One-Hit Theory in the Immune Bactericidal Reaction and Demonstration of a Multi-Hit Response for Hemolysis by Streptolysin O and *Clostridium perfringens* Theta-Toxin

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An analytical method was developed for estimating the number of hits necessary to lyse or kill cells in which various concentrations of the cells are treated with a constant amount of the lytic or killing agent in a constant reaction volume. The reaction may be due to a single-component agent or occur by a sequential chain of reactions due to a multi-component agent, even including side, abortive, or counter-reactions. It was clearly shown by this method that immune bactericidal reactions followed a one-hit theory. It was shown by this method that streptolysin O required four or five hits for hemolysis and *Clostridium perfringens* θ-toxin required two hits. These results were confirmed by both logarithmic dose-response and survival analyses. It was also shown that streptolysin O and θ-toxin can act complementarily on accumulation of the hits for hemolysis.

The one-hit theory of immune hemolysis was developed by Mayer (9, 10) and established through analysis of the kinetics of the C2 reaction step (3). The immune bactericidal reaction has long been suspected to follow this theory but has not yet been confirmed. Since analysis of the single intermediate step of the immune bactericidal reaction is still difficult, analysis of the overall process has been attempted. A theoretical analysis was developed from a reconsideration of Mayer's early experiment in which the absolute number of cells lysed by a limited amount of complement was essentially independent of the sensitized erythrocytes (9, 10).

To test whether this analytical method may be applicable to the multi-hit process, streptolysin O and *Clostridium perfringens* θ-toxin were chosen. These two hemolytic toxins are both oxygen labile and related immunologically (11). Streptolysin O requires multi-hit for hemolysis and does not turn over when it acts on erythrocytes (1, 8). Therefore, these toxins are considered suitable for testing the hit-response analysis. The analytic method developed in this paper can estimate the number of hits necessary for hemolysis by these toxins, which corresponds very well with the value obtained by other methods.

Applying our analytical method, the immune bactericidal reaction was confirmed to follow the one-hit theory.

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THEORETICAL ANALYSIS

In developing a theoretical analysis of the number of hits needed to kill or lyse a certain kind of cells by an agent or group of multi-component agents, the experiment of Mayer was reconsidered, in which the absolute number of cells lysed by a limited amount of complement was found to be essentially independent of the total number of sensitized erythrocytes added (9, 10). For analyzing hits not only derived from a single intermediate step but also resulting from a chain of sequential reactions including abortive formations and decays of some intermediates and effects of some inactivators, it should be suitable to adopt a reaction system in which various concentrations of optimally sensitized bacteria are added to a constant amount of complement in a constant reaction volume. It is also assumed that a complement serum contains a certain number *(N)* of effective sets of components available in a certain reactive system. The effective set of complement components means an effective group of

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the components leading to the formation of a hit on the target cell surface in spite of waste of some part of each component due to abortive inactivation in fluid phase, decay of intermediate sites, or influence of inactivators in the system.

From the Poisson distribution, the proportion of bacteria receiving r effective sets of complement components will be:

$$P_r = \frac{z^r}{r!} e^{-z}$$

If only the bacteria receiving n or more than n hits (n effective sets of complement components) can be killed, the proportion of survivors will be

$$\sum_{r=0}^{n-1} P_r = \sum_{r=0}^{n-1} \frac{z^r}{r!} e^{-z}$$

$$= \sum_{r=0}^{n-1} \frac{\left(\frac{N}{x}\right)^r}{r!} e^{-\frac{N}{x}}$$

$$= 1 - \frac{y}{x}$$

where y is number of killed bacteria, x is the total number of the sensitized bacteria added, N is the total number of effective sets of complement components, and $z = N/x$ is the average number of hits per bacterium.

N will not be the value characteristic of a certain preparation of the lytic or killing agent (e.g., complement serum) and may depend on the different kinds of target cells. N will also be changed theoretically as the number of target cells or of antigen-antibody sites (receptor sites) in the reaction system varies when the lytic agent is extremely labile. Within the experimental conditions mentioned above, however, it can be assumed that N is unchanged regardless of the number of total target cells in a certain reaction system, because the number of the receptor sites on the surface of total target cells can be large enough to react effectively with every complement component or inactivator approaching these sites in a certain reaction system.

In fact, when a varying number of sensitized *Escherichia coli*, which had been labeled uniformly with ^{14}C, were treated with a constant amount of complement, ^{14}C-labeled compounds were liberated into the surrounding medium in proportion to the number of the bacteria, whereas the amount of the phospholipid liberated was constant regardless of the number of the bacteria (to be published elsewhere). Since the phospholipids liberated into the surrounding medium were regarded as related to the lesions in the bacterial surface structure formed by complement, the number of the effective sets of complement components was considered to remain constant even when the number of the bacteria varied in this system.

$$y = x - \sum_{r=0}^{n-1} \frac{N^r}{r!} x^{-(r-1)} e^{-\frac{N}{x}} \tag{1}$$

$$= x\left(1 - e^{-\frac{N}{x}}\right) - \sum_{r=1}^{n-1} \frac{N^r}{r!} x^{-(r-1)} e^{-\frac{N}{x}} \tag{1'}$$

In case of $n = 1$,

$$y = x\left(1 - e^{-\frac{N}{x}}\right) \tag{2}$$

From (1),

$$\frac{dy}{dx} = 1 - \sum_{r=0}^{n-1} \frac{N^r}{r!} \left(\frac{N}{x} - r + 1\right) x^{-r} e^{-\frac{N}{x}} \tag{3}$$

$$= 1 - \left(\frac{N}{x} + 1\right) e^{-\frac{N}{x}} - \sum_{r=1}^{n-1} \frac{N}{r!} \left(\frac{N}{x} - r + 1\right) x^{-r} e^{-\frac{N}{x}} \tag{3'}$$

From (1'), at $x = 0$, $y = 0$.
From (3'),

$$\lim_{x\to 0} \frac{dy}{dx} = \lim_{x\to 0} \frac{\frac{d}{dx}\left\{x^n e^{\frac{N}{x}} - Nx^{n-1} - x^n - \sum_{r=1}^{n-1} \frac{N^r}{r!} (Nx^{n-r-1} - (r-1)x^{n-r})\right\}}{\frac{d}{dx} x^n e^{\frac{N}{x}}}$$

$$= \lim_{x\to 0} \frac{nx^{n+1} - N - e^{-\frac{N}{x}}[(n-1)Nx^n - nx^{n+1}] - \sum_{r=1}^{n-1} \frac{N^r}{r!} e^{-\frac{N}{x}} [(n-r-1)x^{n-r} - (r-1)(x-r)x^{n-r+1}]}{nx^{n+1} - N}$$

$$= \frac{-N}{-N} = 1 \tag{4}$$

$$\lim_{x\to\infty} y = \lim_{x\to\infty} \frac{\frac{d}{dx}\left(1 - e^{-\frac{N}{x}} - \sum_{r=1}^{n-1} \frac{N^r}{r!} x^{-r} e^{-\frac{N}{x}}\right)}{\frac{d}{dx}\frac{1}{x}}$$

Therefore, the slope of the tangent line at the origin should be 45°.
At $x \to \infty$, from (1'),

$$\lim_{x\to\infty} y = N \ (n = 1) \tag{5}$$

$$\lim_{x\to\infty} y = 0 \ (n \geqq 2) \tag{5'}$$

From (3'),

$$\lim_{x\to\infty} \frac{dy}{dx} = 0 \tag{6}$$

Therefore, if x approaches infinity, y will approach N in the case of one-hit killing, but it will approach zero in the case of multi-hit killing. The slope of the curves at $x \to \infty$ should be horizontal regardless of the value for n.

In the case of one-hit killing, the curve should be a simply increasing curve starting at the origin with a tangent line of a slope of 45°, $y = x$, and terminating at $x \to \infty$ into a horizontal line, $y = N$, which corresponds to the "absolute number" of erythrocytes lysed by a certain amount of complement in Mayer's early experiment (9, 10).

In cases of multi-hit killing, the curves also start at the origin with a slope of 45° but terminate at $x \to \infty$ into the axis of the abscissas. Each of them, therefore, should reach a maximal value at the value satisfying $dy/dx = 0$.
From (3'),

$$\left(\frac{N}{x} + 1\right) e^{-\frac{N}{x}} + \sum_{r=1}^{n-1} \frac{N^r}{r!} \left(\frac{N}{x} - r + 1\right) x^{-r} e^{-\frac{N}{x}} = 1 \tag{7}$$

Equation (7) cannot be solved simply except in the case of $n = 1$, where $x \to \infty$, $y \to N$.

Through the courtesy of Y. Tezuka and K. Kaiziri of the Department of Communication Engineering, Faculty of Engineering, Osaka University, the maximum values were calculated by a computer as shown in Table 1.

In addition, the values of y to $x = 0.2N$, $0.3N$, $0.4N$, $0.5N$, $0.75N$, N, $1.5N$, and $2N$ for these curves were also calculated.

The theoretical curves for $n = 1$ to 5 based on these data are shown in Fig. 1.

MATERIALS AND METHODS

Bacterial strain and culture. *E. coli* B/SM, strain 1-1, is a spontaneously occurring mutant strain resistant to streptomycin from *E. coli* B, strain Hershey, as described previously (6). It was maintained on Trypticase soy agar slants (TSB agar slants) (BBL, Cockeysville, Md.) or on Tris(hydroxymethyl)aminomethane (Tris)-glucose medium agar slants like those described by Echols et al. (4) but containing 0.1% glucose instead of 0.2%.

Sheep erythrocytes. Sheep blood mixed with an equal volume of Alsever solution (9) was supplied by the farm at the Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa. The erythrocytes were collected by centrifugation and washed four times with phosphate-buffered saline, pH 6.5 (PBS), or PBS containing 0.005 M ethylenediaminetetraacetate, pH 6.5 (EDTA-PBS), before use.

Media. Physiological saline containing 0.005 M Tris-hydrochloride buffer, pH 7.3, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$ (TBS^{2+}) was used for washing and suspending bacteria and for diluting reagents for the immune bactericidal reaction.

Physiological saline containing 0.046 M phosphate buffer, pH 6.5 (PBS), was used for washing and suspending erythrocytes and for diluting reagents for bacterial hemolysin experiments. When experiments containing a θ-toxin preparation derived from the parent strain (*C. perfringens* PB6K) were carried out, PBS was replaced by a mixture of

TABLE 1. *Maximum values for variables of equation (7)*

n	x	y
1	∞	N
2	$0.5700N$	$0.298423N$
3	$0.3100N$	$0.194167N$
4	$0.2100N$	$0.146995N$
5	$0.7100N$	$0.119522N$

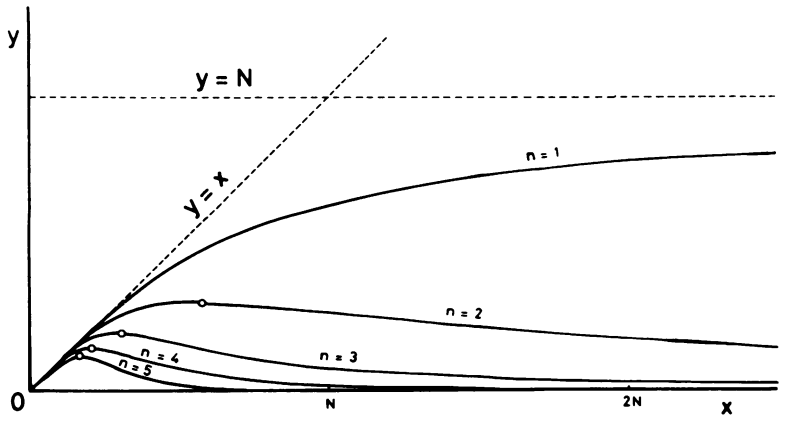

FIG. 1. *Theoretical curves of* $y = x - \sum_{r=0}^{n-1} \frac{N^r}{r!} x^{-(r-1)} e^{-\frac{N}{x}}$. *O, Maximal value of y for each curve.*

19 volumes of PBS and 1 volume of 0.1 M EDTA, pH 6.5 (EDTA-PBS), for inhibiting the effect of α-toxin. In these experiments, the reactions containing streptolysin O were also performed in EDTA-PBS.

Bacterial hemolysins. Streptolysin O was a commercial preparation for the clinical anti-streptolysin O test and was purchased from Nissui Pharmaceutical Co., Tokyo. It was activated as indicated by the manufacturer. One unit of streptolysin O is defined tentatively in this paper as the amount of fully activated toxin in this preparation that is neutralized by 1 Todd unit of international standard anti-streptolysin O antiserum.

Crude θ-toxin of *C. perfringens* was prepared by filtering the culture supernatant of the bacteria grown in buffered fructose proteose peptone medium free from cholesterol (5) through a detergent-free Micro Filter FM-030 (Fuji Photo Film Co., Tokyo). The preparation was either from *C. perfringens* PB6K (the parent strain, $\alpha^+\theta^+$) or from the mixed culture of two of its mutants, MLH9 (group a) and MLH14 (group b), each of which produced no α-toxin and very little θ-toxin (α^-, $\theta^\pm$) but produced a sufficient amount of θ-toxin complementarily and no α-toxin when cultured together (5). Strain PB6K belongs to type A and produces neither δ-toxin nor minor toxin, called non-$\alpha\theta\delta$ hemolysin, which acts on either horse or bovine erythrocytes. When the preparation from the parent strain was used, α-toxin was inhibited by EDTA as described above. The θ-toxin was activated by 0.01 M cysteine at room temperature for 1 h before use.

Antiserum and complement. Rabbits were immunized with heat-killed (56 C for 60 min) *E. coli* B, strain Hershey, that had been cultivated in Tris medium containing 0.1% glucose and then washed. The antiserum was inactivated by heating it at 56 C for 60 min and was then treated three times with bentonite to remove lysozyme activity (7). It was stored at −20 C without any preservative.

Guinea pig complement serum was collected from more than 100 animals. The sera were pooled and centrifuged at 30,000 rpm for 60 min, and the floating lipid layer was removed. Lysozyme-depleted complement serum (RL) was prepared by treating the serum with bentonite as described previously (7). RL was stored at −70 C. The hemolytic activity of RL used was 196 50% hemolytic complement units per ml, estimated by Mayer's standard method (9). Complement was inactivated by heating it at 56 C for 60 min.

Estimation of the immune bactericidal reaction. The bacteria were cultivated in Tris medium containing 0.1% glucose at 37 C for 13 to 16 h with constant shaking. They were collected by centrifugation and washed twice with TBS^{2+}. They were resuspended in TBS^{2+} at a concentration of 1.0×10^9 bacteria/ml. The suspension was mixed with an equal volume of lysozyme-depleted antiserum diluted also in TBS^{2+} in the cold. In case of suboptimal sensitization, a 1:10 dilution of antiserum for optimal sensitization was used. The mixture was transferred into a 37 C bath and incubated for 15 min with constant shaking. After incubation, the bacteria were collected by centrifugation and washed twice with TBS^{2+}. They were resuspended and diluted in TBS^{2+} to give the number of sensitized bacteria necessary to each reaction tube. One milliliter of each suspension was mixed with 1.5 ml of a dilution of RL in TBS^{2+} and incubated at 37 C for 60 min with constant shaking. As a control, a similar system including heat-inactivated RL, instead of RL, was included. After incubation, each reaction mixture was diluted serially in cold TBS^{2+}. A 0.1-ml amount of the dilution was overlayed with 5 ml of soft TSB agar, which had been melted and kept at about 48 C, on a TSB agar plate containing 250 μg of streptomycin per ml. After solidification, the plates were incubated at 37 C overnight. The number of killed bacteria was calculated from the difference between colony counts from the control system and those from the RL system.

Estimation of hemolysis. The reaction mixtures were set up in an ice bath. They were then transferred into a 37 C bath and incubated at 37 C for 30 min. After incubation they received 2 volumes of cold PBS and were centrifuged. Controls without hemolysin for spontaneous lysis and with distilled water for complete lysis were also included. Optical density of the supernatant was read at 541 or 413 nm, depending on the cell concentration used. When erythrocytes were treated with a sublytic concentration of hemolysin, they were prepared by incubation at 37 C for 30 min followed by washing four times with PBS or EDTA-PBS at room temperature.

Computer. The values of x and y for maxima and those of y to $x = 0.2N$, $0.3N$, $0.4N$, $0.5N$, $0.75N$, N, $1.5N$, and $2N$ for theoretical curves of $n = 1$ to 5 were calculated by an NEAC computer, model 3200 (Nippon Electric Co., Tokyo), through the courtesy of Y. Tezuka and K. Kaiziri of Osaka University.

RESULTS

Cumulative hits necessary for hemolysis by streptolysin O and C. perfringens θ-toxin. A suspension of washed erythrocytes was divided into two portions, one of which was mixed with a sublytic concentration of streptolysin O and another with buffer (PBS or EDTA-PBS). They were incubated at 37 C for 30 min. They were then centrifuged and washed thoroughly at room temperature and resuspended in the buffer. Each of these suspensions was divided further into two series; one received a varying amount of streptolysin O, and the other received θ-toxin. They were incubated again at 37 C for 30 min.

Lysis of streptolysin-pretreated erythrocytes by either streptolysin O or θ-toxin was greater than that of nontreated erythrocytes, i.e., those preincubated with buffer, by each toxin (Fig. 2). Therefore, it is obvious that the hits formed by these toxins are cumulative and that these toxins act complementarily on hemolysis. A similar effect of each toxin was also observed with θ-toxin-pretreated erythrocytes.

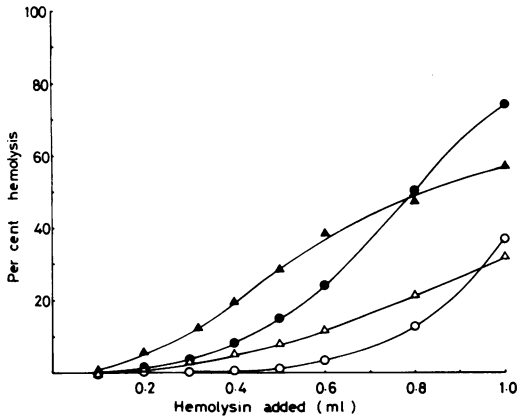

FIG. 2. *Lysis of streptolysin O-treated and non-treated erythrocytes by further treatment with streptolysin O or θ-toxin. A suspension of erythrocytes at a concentration of 4.0 × 10⁹ cells/ml in EDTA-PBS was mixed with an equal volume of the same buffer (○, △) or 0.2 U of streptomycin O per ml in EDTA-PBS (●, ▲). Both kinds of the cells were centrifuged, washed four times with EDTA-PBS, and resuspended in the same buffer at a concentration of 1.0 × 10⁹ cells/ml. One milliliter of each suspension received 0.2 U of streptolysin O per ml (○, ●) or a 1:1,000 dilution of crude θ-toxin (△, ▲) in the amount indicated on the abscisssa. The reaction was carried out in a volume of 2.0 ml at 37 C for 30 min.*

When the logarithm of the proportion of cells lysed was plotted versus the logarithm of the amount of toxin, the dose-response curves shown in Fig. 2 could be converted to straight lines (Fig. 3). In this experiment, the slope of the line for streptolysin O lysis of untreated cells was 5, whereas that for θ-toxin lysis was 2; that is, the fraction of lysed erythrocytes was proportional to the fifth power of the amount of streptolysin O or to the second power of the amount of θ-toxin. Therefore, streptolysin O requires five hits for hemolysis and θ-toxin requires two (12). Similar experiments showed four or five hits or values between them for streptolysin O lysis and two or three hits for θ-toxin lysis regardless of the buffer used (PBS or EDTA-PBS). Moreover, θ-toxin derived either from the parent strain or from the mixed culture of both groups (a and b) of the α^- $\theta^\pm$ mutants (5) behaved similarly.

In the experiment shown in Fig. 2 and 3, the slopes for streptolysin O-pretreated cells were 3 to streptolysin O and 1.7 to θ-toxin.

Similar experiments using rabbit erythrocytes instead of sheep cells also showed that about four hits were necessary for the lysis by streptolysin O.

Survival analysis of hemolysis by streptolysin O. Hemolysis by streptolysin O was further analyzed in a wider range of degree of lysis. If the logarithm of the fraction of nonlysed erythrocytes was plotted versus the amount of streptolysin O, a typical multi-hit survival curve was obtained (Fig. 4A). If the linear portion of the curve was extrapolated to the axis of the ordinates, a value of 5 was obtained in this experiment. If the same experiment was expressed by logarithmic dose-response plotting as in Fig. 3, a curve was obtained (Fig. 4B). The lower linear portion of the curve also showed a slope of 5. Therefore, both analyses showed that hemolysis by streptolysin O required five hits in this experiment also.

When the experiment was expressed by logarithmic von Krogh plotting (9), the plots for the higher degree of hemolysis gave a straight line (Fig. 4C). A value of 0.263 was obtained for the slope ($1/n$ in the von Krogh equation) of this line.

Analysis of lysis of varying concentration of erythrocytes by a constant amount of streptolysin O or θ-toxin. The experiment shown in Fig. 5 was carried out at the same time, by using the same materials used in the experiment shown in Fig. 1 and 2, by the analytical method using varying concentrations of erythrocytes with a constant amount of hemolytic toxin in a constant reaction volume. The hemolytic curves for three different concentrations of streptolysin O corresponded well with the theoretical curves of $n = 5$ or 4; those for θ-toxin also corresponded well with curves of $n = 2$.

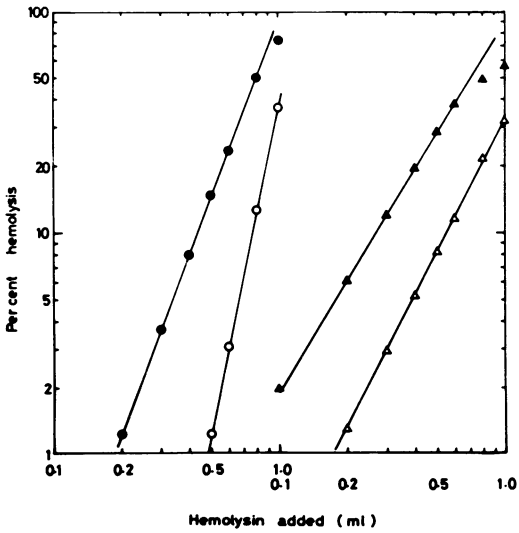

FIG. 3. *Lysis of streptolysin O-treated and non-treated erythrocytes by further treatment with streptolysin O or θ-toxin. Logarithmic plotting of the experiment shown in Fig. 2.*

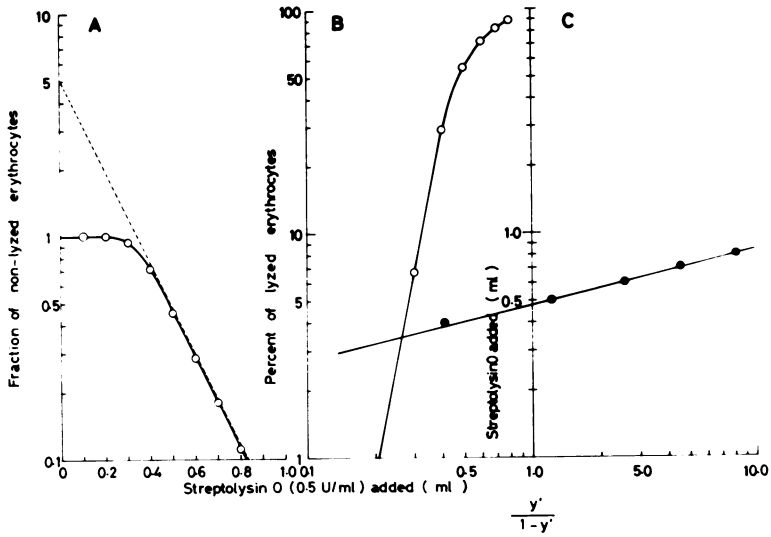

FIG. 4. *Hemolysis by streptolysin O. Streptolysin O at a concentration of 0.5 U/ml in the volume indicated on the abscissa was added to 1.0 × 10^9 erythrocytes in a final volume of 2.5 ml. The reaction was carried out at 37 C for 30 min. Medium used was PBS. y' indicate the degree of hemolysis.*

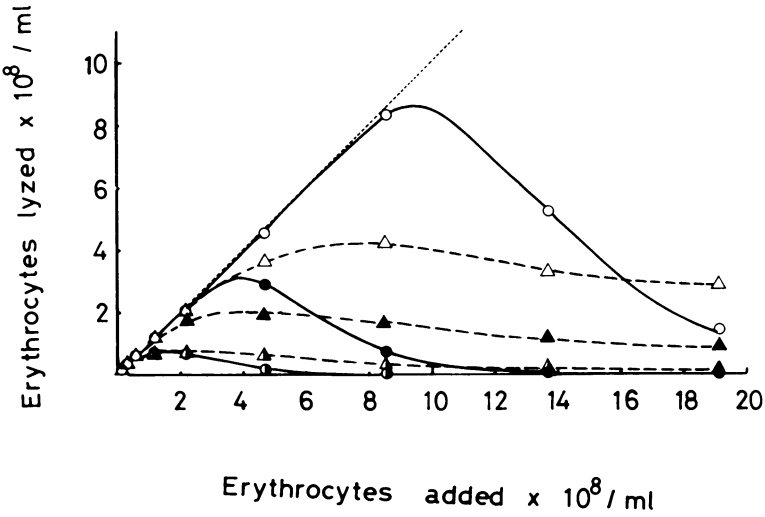

FIG. 5. *Lysis of varying concentrations of erythrocytes by a constant amount of streptolysin O or θ-toxin. Erythrocytes at the final concentration indicated on the abscissa were incubated with streptolysin O at a final concentration of 0.2 (○), 0.1 (●), or 0.05 (◑) U/ml or θ-toxin at a dilution of 1:1,000 (△), 1:2,000 (▲), or 1:4,000 (◭) at 37 C for 30 min.*

Analysis of hit response in the immune bactericidal reaction. When various concentrations of optimally sensitized bacteria were treated, in a constant reaction volume, with a certain amount of complement, a killing curve like that shown in Fig. 6 was obtained. It corresponds very well with the theoretical curve of $n = 1$. Therefore, we conclude that the immune bactericidal reaction proceeds in a one-hit killing process similar to immune hemolysis.

DISCUSSION

Alouf and Raynaud reported that about 360 molecules of streptolysin O were required to lyse one erythrocyte (2). They (1) also showed that two hits were necessary for hemolysis from

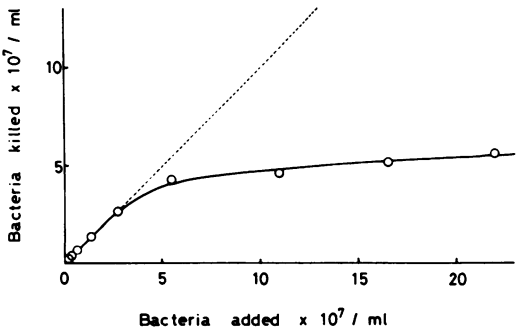

FIG. 6. *Immune bactericidal reaction. Optimally sensitized bacteria at the final concentration indicated on the abscissa were incubated at 37 C for 60 min with RL or inactivated RL at a final concentration of 1:250. See text for details.*

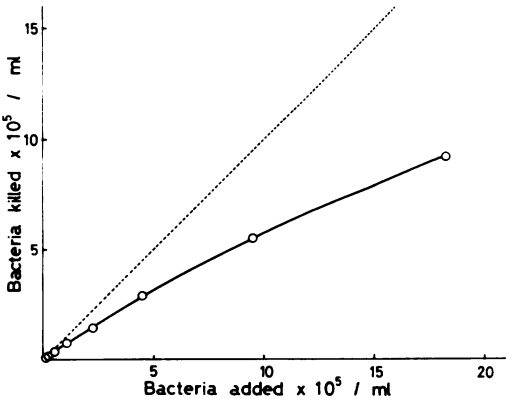

FIG. 7. *Immune bactericidal reaction with poorly sensitized bacteria. Suboptimally sensitized bacteria were treated as in the experiment in Fig. 6 with RL or inactivated RL at a final concentration of 1:500. Concentrations of bacteria were chosen to be much lower than those used in Fig. 6 for emphasizing the complement-excess region. See text for details.*

the extrapolation of linear portions of survival curves similar to that shown in Fig. 4, in which a value of 5 was obtained. This type of survival analysis is very suitable to experiments such as ultraviolet light inactivation of bacteriophage, in which survivors can easily be pursued to a magnitude of 10^{-3} or 10^{-4} of the original input. However, it is very difficult to measure exactly 99.9 or 99.99% hemolysis for obtaining the real linear portion of the curve. Kanbayashi et al. (8) also reported that streptolysin O required about two hits for lysis of rabbit erythrocytes. However, their theoretical analysis seems to rest on oversimplified and conflicting assumptions.

The logarithmic dose-response analysis of the lower-degree hemolysis (Fig. 3) also gave a value of 5 or 4 for the hits required for the hemolysis of streptolysin O. A value of about 4 was also obtained for streptolysin O-mediated lysis of rabbit erythrocytes, which were used for the target cells by Alouf and Raynaud (1) and by Kanbayashi et al. (8).

C. perfringens θ-toxin is known to be related to streptolysin O, pneumolysin, and tetanolysin; these toxins are all oxygen labile and cross-neutralized immunologically (11). Moreover, θ-toxin and streptolysin O can act complementarily on accumulating hits for hemolysis (Fig. 2 and 3). Two and sometimes three hits are necessary to lyse one erythrocyte by θ-toxin.

These experiments showed clearly the multi-hit process of hemolysis mediated by these toxins. The analysis using various concentrations of erythrocytes and a constant amount of toxin also agrees well with the results mentioned above. The shape of the hemolytic curves (Fig. 5) corresponds with the theoretical curve of $n = 5$ or 4 in case of streptolysin O and with $n = 2$ for θ-toxin.

When this new analytical method is applied to the immune bactericidal reaction, a one-hit killing curve is obtained (Fig. 6). Therefore, we conclude that complement can kill a bacterium by a single hit.

This analytical method is simple and applicable to the single-component killing or lytic agent as well as the overall process of sequential reactions by a multi-component agent, even when including side, abortive, or counter-reactions.

This analytic method has been developed from the assumption that the bacteria are optimally sensitized; i.e., they possess enough antigen-antibody sites. If the target cells have only a few receptor sites for the hit formation, the shape of the curve will be depressed toward the axis of the abscissas. In the case of immune cytolysis, this can result from a lack of sufficient antigenic sites per cell or from insufficient sensitization. Figure 7 shows results obtained by using suboptimally sensitized bacteria, in which the slope of the curve near the origin is less than 45°, showing that some fraction of bacteria survive even in the complement-excess region.

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LITERATURE CITED

1. Alouf, J. E., and M. Raynaud. 1968. Action de la streptolysine O sur les membranes cellulaires. Ann. Inst. Pasteur Paris 115:97-121.
2. Alouf, J. E., and M. Raynaud. 1968. Some aspects of the mechanism of lysis of rabbit erythrocytes by streptolysin O, p. 192-206. *In* R. Caravanco (ed.), Current research on group A streptococcus. Excerpta Medica Foundation, Amsterdam.
3. Borsos, T., H. J. Rapp, and M. M. Mayer. 1961. Studies on the second component of complement I. The reaction between EAC'1,4 and C'2: evidence on the single site mechanism of immune hemolysis and determinaton of C'2 on a molecular basis. J. Immunol. 87:310-325.
4. Echols, H., A. Garen, S. Garen, and A. Torriani. 1961. Genetic control of repression of alkaline phosphatase in *E. coli*. J. Mol. Biol. 3:425-438.
5. Higashi, Y., M. Chazono, K. Inoue, Y. Yanagase, T. Amano, and K. Shimada. 1973. Complementation of θ-toxigenicity between mutants of two groups of *Clostridium perfringens*. Biken J. 16:1-9.
6. Inoue, K., A. Takamizawa, K. Yano, and T. Amano. 1974. Chemical studies on damages of *Escherichia coli* by the immune bactericidal reaction. I. Release and degradation of phospholipids from damaged bacteria. Biken J. 17:127-134.
7. Inoue, K., Y. Tanigawa, M. Takubo, M. Satani, and T. Amano. 1959. Quantitative studies on immune bacteriolysis. II. The role of lysozyme in immune bacteriolysis. Biken J. 2:1-20.
8. Kanbayashi, Y., M. Hotta, and J. Koyama. 1972. Kinetic study on streptolysin O. J. Biochem. 71:227-237.
9. Mayer, M. M. 1961. Complement and complement fixation, p. 133-240. *In* E. A. Kabat (ed.), Kabat and Mayer's experimental immunochemistry, 2nd ed. Charles C Thomas, Publishers, Springfield, Ill.
10. Mayer, M. M. 1961. Development of the one-hit theory of immune hemolysis, p. 268-279. *In* M. Heidelberger and O. J. Plescia (ed.), Immunochemical approaches to problems in microbiology. Rutgers University Press, New Brunswick, N.J.
11. Todd, E. W. 1941. The oxygen-labile haemolysin or θ toxin of *Clostridium welchii*. Br. J. Exp. Pathol. 22:172-178.
12. Webber, B. B., and F. J. de Serres. 1965. Induction kinetics and genetic analysis of X-ray-induced mutations in the ad-3 region of *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S.A. 53:430-437.